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Review

Why Should Growth Hormone (GH) Be Considered a Promising Therapeutic Agent for Arteriogenesis? Insights from the GHAS Trial

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Abstract: Despite the important role that the GH/IGF-I axis plays in vascular homeostasis, these kind of growth factors barely appear in articles addressing the neovascularization process. Currently, the vascular endothelium has turned to be considered as an authentic gland of internal secretion due to the wide variety of released factors and functions with local effect, including the paracrine/autocrine production of GH or IGF-I, for which the endothelium has specific receptors. In this comprehensive review, it will be described the evidence involving these proangiogenic hormones in arteriogenesis dealing with the arterial occlusion and making of them a potential therapy. It will be analyzed all those elements triggering the local and systemic production of GH/IGF-I and their possible role both in physiological and pathological conditions. The whole evidence will be combined with important data from the GHAS trial, in which GH or placebo were administrated to patients suffering from critical limb ischemia with no option for revascularization. We postulate that GH, alone or in combination, should be considered as a promising therapeutic agent for helping in the approach of the ischemic disease.

Keywords: GH and eNOS; IGF-I; oxidative stress and arterial inflammation; vascular homeostasis; Neovascularization; arteriogenesis; GHAS trial

1. Introduction

The fact that growth hormone (GH) is a necessary actor for many physiological processes and a real breakthrough for the treatment of many pathological situations beyond simple human longitudinal growth currently needs no justification [1]. Today, GH is considered a key hormone that acts in virtually all organs and tissues in which it performs important specific functions. From the current knowledge of the GH actions can be inferred that, overall, this hormone is a hormone for cell proliferation and survival. The persistent GH secretion out of the growth period is a clear proof of the importance of the actions of this hormone at multiple levels as the cardiovascular, hematopoietic and immune systems, among others [1].

In fact, virtually all organs and tissues have receptors for this endocrine hormone, and the hormone is also produced in practically all the cells of the organism where it plays specific autocrine/paracrine roles [1]. However, the sometimes-contradictory results of the use of GH in clinical trials only reflect how little we know about how this hormone really works and how dependent it is on the physiological or pathological state and the microenvironment in which it is acting or the dose or time during which GH is administered. Many evidences support the participation of local or circulating GH in vascular homeostasis, since when this hormone lacks endothelial dysfunction appears with severe consequences; most likely this is the reason by which untreated GH-deficiency is associated with and increased risk of atherosclerosis and vascular mortality, while GH treatment may reverse early atherosclerosis [2–5]. A recent study in subjects without GH-deficiency (GHD) or any cardiovascular disease (CVD), but with one or more CV risk factors (age, smoking, obesity, hypertension, dyslipidemia, insulin resistance), demonstrated that GH and its mediator IGF-I play a protective role in arterial wall changes associated with vascular aging [6]. In fact, receptors for GH (GHR) and IGF-I (IGF-IR) are expressed in the vascular endothelium [7–9], and some studies suggest that GH itself is expressed in this special gland of internal secretion [10,11]. These data indicate that GH and IGF-I have to play a very important role in the maintenance of a normal endothelial function. Endothelial dysfunction in GHD was demonstrated by an impaired flow-mediated dilation, which improved with GH treatment [12], indicating that GH had to play a role in vascular reactivity [13], as it had been shown by the group of Napoli [14]. Besides it, GH treatment in GHD patients also leads to the normalization of the high arterial wall thickness and arterial stiffness in these patients [5], and normalizes a series of markers of endothelial dysfunction that are generally increased in untreated GHD patients [15]. This brief introduction allows to understand the very important role that GH plays in the cardiovascular system as it has been reviewed in several occasions [9,16,17]. Deleterious changes in arteries with aging influence negatively on the capacity of compensation after arterial occlusion [18], something that it has to be highlighted that occurs parallelly with the GH decline experienced as we get older [1,19,20]. As is known, redox imbalance during aging is responsible for this negative effect on arteries, and GH, as it will be demonstrated during the text, has many to do.

In this review, we will analyze those aspects about how GH and its mediator IGF-I can act in the arterial wall favoring the normal physiologic functioning, and how both molecules play an important role in collateral remodeling after the arterial occlusion. In addition, we will bring to light some surprising data that may have been overlooked so far in arteriogenesis, with the aim of improving the understanding, not only of the typical role attributed to GH in the induction of eNOS and the production of NO, but also some ideas about the role of the redox system in the control of homeostasis and vascular remodeling or to clarify how vessels respond to shear stress forces (SSF) to increase their final size with the participation of the GH / IGF-I system. Step by step, arteriogenesis will be sheared underlining those aspects in which GH could help. Finally, in an attempt to make a leap from bench to bedside, as a novelty we will present some molecular data obtained from the GHAS trial on the benefit of using GH as a rescue therapy in real patients with critical limb ischemia without options for conventional revascularization.

2. Vascular homeostasis: role of the GH/IGFI axis

A normal embryonic development needs the formation of blood vessels [21]; after birth there is also the need of the formation of new blood vessels while growing, but also in some physiological processes such as the menstrual cycle in women and the development of the mammary gland during pregnancy [22], but apart from these situations, adult neovascularization rarely takes place and when it happens it is associated with pathological affectations, such as wounds, muscle injuries, fractures or hypoxia/ischemia. The question now would be: how does neovascularization occur in adults and what is the role of the GH / IGF-I system in it?

As it seems logical, hypoxia is a very important stimulus for the growth of new blood vessels [23], triggering this growth through hypoxia-inducible factors (HIF) that act on the expression of pro-angiogenic factors, but also that of anti-angiogenic factors to achieve the perfect number and size of the vessels necessary to compensate for the lack of oxygen supply and to regenerate the tissue [23]. This is a clear and well-established fact for angiogenesis. However, when a progressive narrowing of the vascular lumen appears, preexisting collaterals will have to growth to compensate the lack of distal flow which is triggered in a totally different way. With independence of that, the stimulation of endothelial nitric oxide synthase (eNOS) that leads to the production of nitric oxide (NO) from the vascular endothelium seems to be the key mediator for both kind of reparative processes. NO is a potent vasodilator, therefore increasing blood supply to the zone affected by hypoxia/ischemia. This implies changes in the vascular tone, vasorelaxation and vasopermeability, which are affected in arteries suffering an atherosclerotic damage. Interestingly, GH activates the NO pathway [14,24] throughout direct mechanisms that seem to be specific and independent of the GH-mediator IGF-I [24], although ancient studies indicated that elevated plasma levels of IGF-I increase NO release in cultured endothelial cells (ECs) [25,26], and more recent data show that both GH and IGF-I regulate the expression of eNOS in the aorta of hypophysectomized rats [27]. At this point it seems to be of interest to indicate that the gastric GH-secretagogue ghrelin has been shown to induce vasorelaxation by stimulating eNOS expression in GHD rats [28]. These and many other studies clearly show that members of what we might call the GH system (GH itself, IGF-I, GH-secretagogues and inhibitors of GH-signaling pathways) play a key role in the vascular homeostasis.

The next question could be: how does GH act at this level?

Endocrine GH interacts with its membrane receptor (GHR) and activates the associated JAK2 (Janus kinase 2) and Src family kinases, leading to a cascade of tyrosine phosphorylation. Contrary what was been thought for years, the activation of the GHR leads to movements within a receptor homodimer, rather than simple receptor dimerization. These GHR movements produce the separation of the two associated JAK2s, and the removal of an inhibitory pseudokinase domain from the kinase domain of the other JAK2 (and vice versa). The result of this is that the two kinase domains are put in position for trans-activation and initiate tyrosine phosphorylation of the cytoplasmic receptor domain, which leads to the phosphorylation of a series of signaling pathways, such as the factors of transcription STATs (Signal Transducer and Activator of Transcription), particularly STAT5 responsible for mediating most of the effects of GH at the genomic level, and especially involved in GH-induced cell proliferation [for a more detailed explanation see [29,30], and postulated as responsible of eNOS activation [31], although this has not been demonstrated. Other key signaling pathway activated by tyrosine phosphorylation after the interaction GH-GHR is that of PI3K/Akt (phosphoinositide 3-kinase/serine-threonine kinase), stimulated after activation by JAK2 of the Insulin receptor substrate (IRS); the PI3K/Akt pathway is involved in cell survival [32], because it inhibits proapoptotic caspase 3 [33], but it is also an inducer of eNOS activation and NO production [31]. This enhanced eNOS is not exclusively dependent of GH, since IGF-I also induces Akt phosphorylation leading to activation of eNOS [34], although it is unlikely that this effect of IGF-I is due to the induction by GH of the endothelial expression of IGF-I [8]. Moreover, the effects of GH on vascular production of NO seem to be also independent of GH-induced plasma IGF-I [14], somewhat quite contradictory given the effects just described of GH and IGF-I in the production of endothelial NO. Another important signaling pathway activated by GH is the Shc adapter proteins, because they induce the activation of the Grb2-SOS-Ras-Raf-ERK (extracellular signal-regulating kinase) pathway [35]; activated ERK translocates into the nucleus and regulates the expression of genes involved in

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cell proliferation, differentiation and survival, but also, and perhaps more important, regulates cell motility and migration [36], a mechanism very important for the formation of new vessels as we will describe later. The interaction GH-GHR also induces the activation of the focal adhesion kinase (FAK), responsible for the reorganization of the cytoskeleton in many cell types [37,38], although its effects on vascular ECs have not yet been demonstrated. These concepts are schematized in Figure 1.

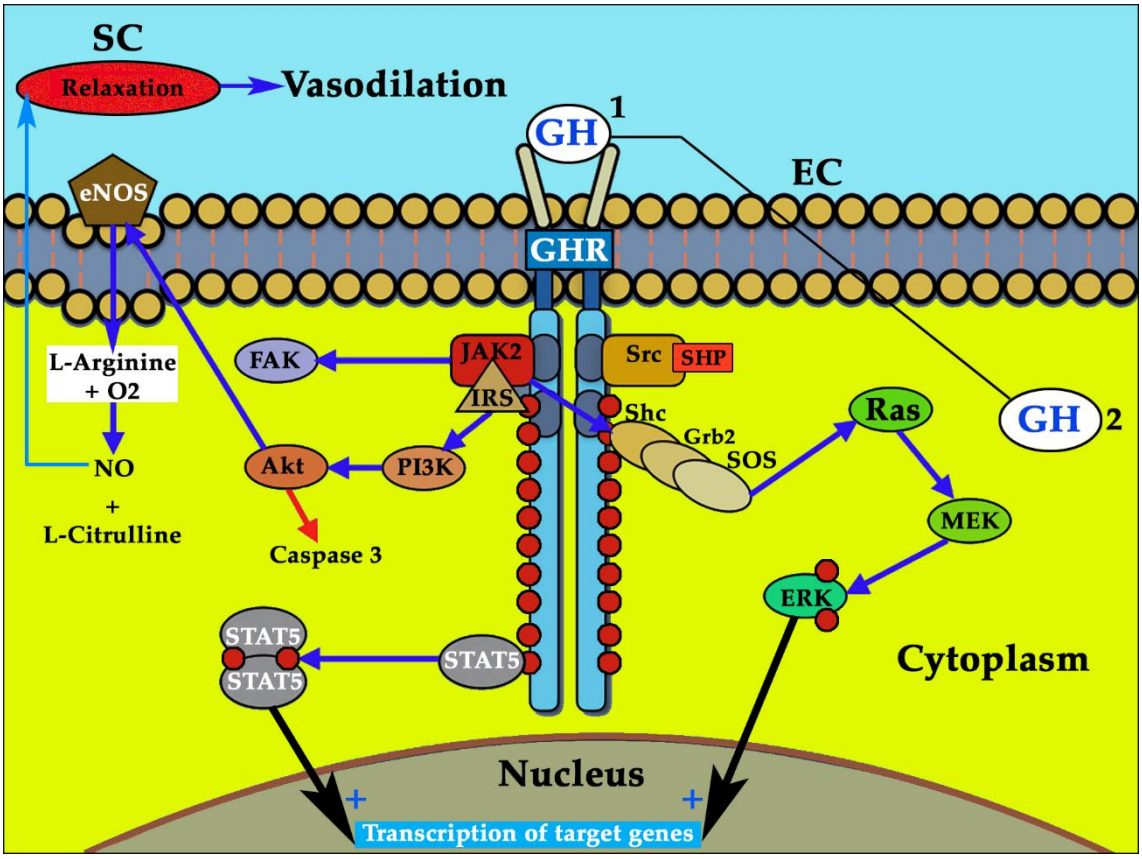

Figure 1. Effects of GH on the vascular endothelium.
The interaction GH-GHR produces the activation of the associated Janus Kinase 2 (JAK2), which induces the phosphorylation (red circles) of tyrosine located in the cytoplasmic receptor domain, leading to the phosphorylation of GH-signaling pathways, such as STATS. Among STATS (STATS 1 and 3 not shown in the Figure), STAT5 homodimerizes and is translocated to the nucleus where it induces the transcription of a series of genes. Activated JAK2, acting on the Insulin receptor substrate (IRS) induces the phosphorylation of PI3K which, in turn, activates the cell survival factor Akt. This inhibits the proapoptotic enzyme Caspase 3 (red arrow), but also activates eNOS (blue arrow). Activated ENOS promotes the synthesis of NO (from L-Arginine + O2) and the formation of L-Citrulline. The NO formed flows from the cytoplasm to the muscle cell layer of the blood vessels, producing its relaxation and consequent vasodilation. The interaction GH-GHR also induces the activation of the Shc adapter proteins, which leads to the activation of Grb2-SOS-Ras-Raf-MEK-ERK pathway (Raf is not shown in the Figure). Activated ERK translocate into the nucleus of the ECs and regulates the expression of genes involved in cell proliferation, differentiation and survival, but also regulates cell motility and migration (key for the formation of new vessels). GH-GHR interaction also activates FAK. SHP: Protein tyrosine phosphatase. Blue arrows: stimulation. Red arrow: inhibition. +: stimulation. 1: Endocrine GH. 2: Endothelial GH: plays and auto/paracrine role and in situations of absence of endocrine GH perhaps plays the role of the former (black line).

Given the effects of GH on the production of endothelial NO, it could be expected that GH-increased NO could induce a toxic effect on the vascular endothelium in situations of increased oxidative stress, since in these situations NO can be eliminated by superoxide (O2-) leading to the

production of peroxynitrite (ONOO-), which is a strong toxic to the endothelium [39,40], and hydroxyl radical OH(*), termed highly reactive oxygen species (hROS). This is what happens in patients with GHD, but it is normalized after replacement therapy with GH [13]. This is in agreement with previous studies that demonstrate that GH has a protective effect on mitochondria [41,42], which are the main source of oxidants within cells and a main objective of oxidative stress, but also produce elimination systems of oxidants. This is the reason why mitochondrial dysfunction leads to a greater generation of hROS which, in turn, contributes to the presentation of a senescent phenotype in ECs and to the activation of redox-sensitive transcription factor NF-kß [43], that decreases endothelial-induced vasodilation and increases the expression of inflammatory genes in the vasculature of aging rats and humans [44,45]. However, as indicated above, plasma physiological levels of GH and IGF-I modify intracellular levels of oxidative stress [13,46,47]. These concepts are schematized in Figure 2.

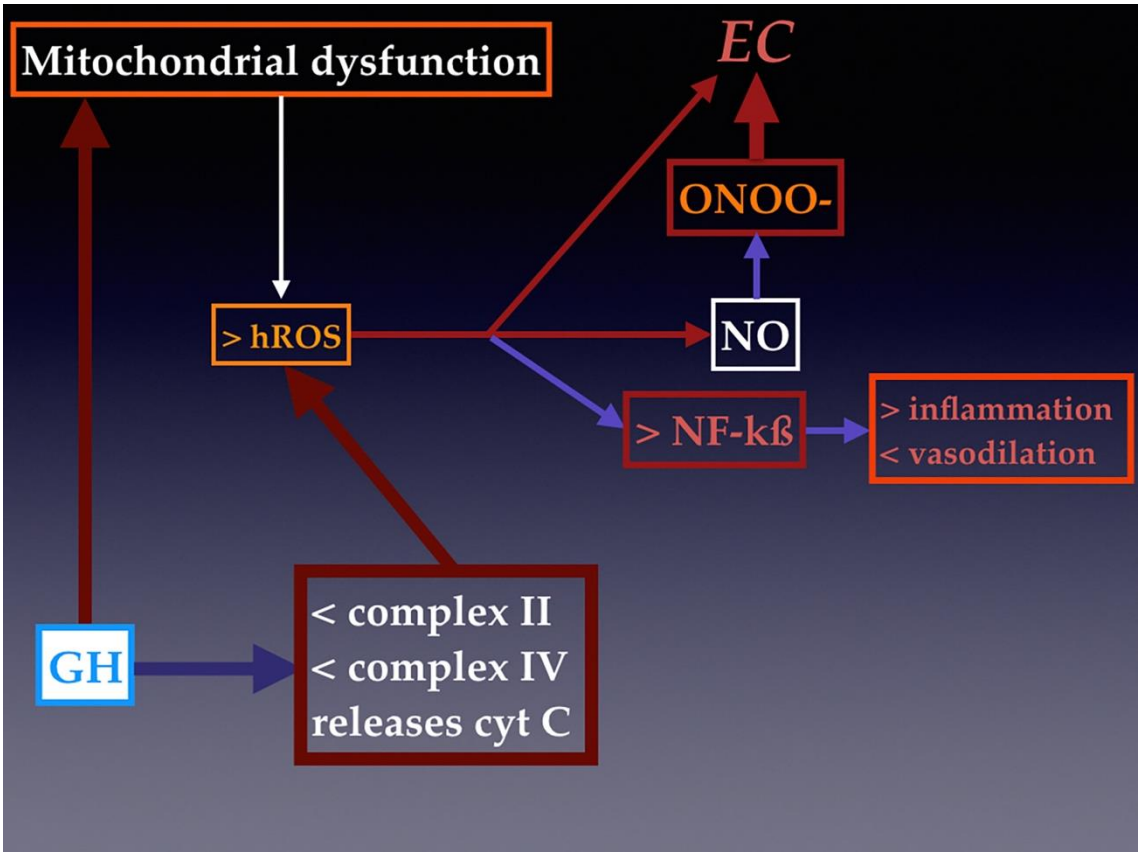

Figure 2. Mitochondrial dysfunction leads to vascular endothelium senescence.
An excessive production of hROS by mitochondria induces the elimination of NO, because it is transformed into peroxynitrite (ONOO-), toxic to the endothelial cells. Moreover, the excessive oxidative stress induces the activation of NF-kß, which increases the expression of inflammatory genes in the blood vessels and decreases vasodilation. These lead to the presentation of a senescent phenotype in the endothelial cells. GH administration corrects mitochondrial dysfunction, because GH is able to enter to mitochondria and decrease the activity of complexes II and IV of the mitochondrial respiratory chain. In addition, GH produces a decrease in the mitochondrial membrane potential which translates into the release of cytochrome C (cyt C) to the cytosol. Blue arrows: stimulation. Red arrows: inhibition or damage (in endothelial cells).

GH is capable of translocating to mitochondria [48,49], and it has been shown that in isolated mitochondria high concentrations of GH can diminish the production of mitochondrial O2-, most likely by inducing a decrease in the activity of complexes II and IV of the mitochondrial respiratory chain [49], by modulating the mitochondrial membrane potential critical for maintaining the

physiological function of the respiratory chain to generate ATP, because when this membrane potential collapses cytochrome C is released in the cytosol and therefore the mitochondrial respiratory chain is affected (Figure 2).

Hence, it is clear that GH plays a positive role in homeostasis of the vascular endothelium, not only by increasing NO production but also by protecting the endothelium, acting at the mitochondrial level, from oxidative stress. However, it has been described that this effect of GH does not occur acutely, but after a time, at least in studies performed in vitro [7,50].

From these data, it is tempting to hypothesize that the progressive decrease in GH secretion that occurs during aging is a factor involved in mitochondrial dysfunction, oxidative damage and endothelial dysfunction.

Since, as indicated above, GH is expressed in ECs, where it plays an autocrine / paracrine role, one might think that the loss of endocrine secretion of GH as we age could be compensated with the cellular expression of this hormone, but this has been seen only in mammary carcinomas [11], in which endothelial GH stimulates the proliferation, migration, survival and capillary formation of ECs. Hence, there is no data to prove the possibility of an effect of autocrine/paracrine endothelial GH substituting the effects of endocrine GH in normal subjects. In addition, as far as we know, it is unknown how GH expression is regulated in vascular ECs, and if the decrease in endocrine production of this hormone, in old people for instance, is accompanied by a deficit in its cellular production, although a GHRH-GH axis has been demonstrated to exist in retinal neurons [51,52].

The effects of GH on vascular homeostasis are not limited to the induction of NO production and its protective effects on mitochondria and oxidative stress. The hormone may also increase vascularization by stimulating the release of bone marrow endothelial precursor cells (EPCs) into the blood, an effect seen in healthy adults [53,54], and also in elder people after administration of GH [55]. The effect of EPCs on the maintenance and repair of blood vessels has been proposed many years ago [56,57]. They are released in response to ischemia for increasing neovascularization of ischemic tissues. Among these EPCs, CD34+ cells appear to be the most important at the functional endothelial level, as it has been demonstrated in GHD adults after being treated with GH [58]. In fact, GH is a strong inducer of the production and release of EPCs, as it has been demonstrated not only in healthy volunteers [53,54], elder people [55], and GHD adults [58], but also in patients with relapsed or refractory hematologic malignancies, in which after a myeloablative therapy there is a need to induce a rapid hematopoietic recovery; in these patients it has been observed that GH mobilizes CD34+ cells efficiently [59]. CD34+ cells differentiate to ECs, so they increase both the stabilization of blood vessels and the rapid development of these vessels, therefore CD34+ cells are very useful for the treatment of wounds and ischemic disorders [60]. Interestingly, CD34+ cells express GH and IGF-I receptors [61].

Another important effect of GH is that exerted on Mesenchymal Stem Cells (MSCs). These cells have been thought to proceed from the bone marrow, where they were identified in the past century [62], but we currently know that they can be found in virtually all adult tissues [63–65], particularly in the adipose tissue where they seem to be located in a perivascular zone near to pericytes and ECs [66]. In fact, it has been proposed that blood vessels possess MSCs in their perivascular niche [67]. This is very important since the external layer of blood vessels, also called tunica adventitia, that has long been relegated as a mere support of the tunica media, it is currently known that participates in vascular remodeling, since its cells can be activated in response to injuries [68–70], hypoxia [71], and hypertension [72]. Although the activation of adventitial progenitors mainly results in proliferation and differentiation into myofibroblasts that migrate into the inner layers of the vascular wall, and release of paracrine factors which regulate vascular remodeling [73]. Multipotent progenitors showing a MSCs phenotype have been isolated from the tunica adventitia of the pulmonary artery in humans [74], expressing MSCs surface markers (CD34+ CD31- CD146-) [67]. This agrees with previous studies demonstrating that MSCs can promote neovascularization and endothelium repair [75]. Therefore, although the effects of GH on MSCs, well known at the adipose tissue level, mainly have been described as an inhibitory effect on MSCs differentiation towards adipocytes [76], it is likely that at the vascular level GH induces the differentiation of these cells to ECs. At this point, it is

of interest to indicate that MSCs differentiate to ECs in the presence of the vascular endothelial growth factor A (VEGF-A) [77,78], a factor that plays many roles in cell differentiation, proliferation and angiogenesis. The relationships between GH and VEGF-A are not well known, but GH plays a pivotal role among the factors that regulate VEGF family expression in humans [79]. On the other side, GH regulates the expression of different genes involved in Notch-1 signaling, at least at the ovarian level [79], and two Notch-1 ligands, delta-like protein Dl14 and Jagged 1 regulate angiogenesis directly in the endothelium [80,81], but besides it there is a strong relationship between VEGF-A and Notch-1 signaling. Therefore, given the relationships existing between Notch-1 and VEGF-A, and Notch-1 and GH, it is presumable that the differentiation of MSCs to ECs, neovascularization and endothelium repair, may involve the participation of GH, Notch-1 and VEGF-A [for a more detailed explanation, see Figure 9 in reference 79].

As is logical, so that vascular homeostasis is fulfilled correctly, negative regulation mechanisms must exist to prevent an excess of intracellular signaling by GH after its interaction with its membrane receptor. The negative regulation of GH signaling is mainly performed by intracellular protein tyrosine phosphatases 1B and H1, suppressors of cytokine signaling (SOCS, 1, 2 and 3), sirtuin 1, protein inhibitors of activated STAT (1, 3 and 4), cytokine-inducible SH2-containing protein (CIS), and Src homology 2 (SH2) domain containing protein tyrosine phosphatase (SHP) [82] (Figure 1). Given the different systems that act on the negative regulation of GH signaling pathways, there must be a perfect balance between them to prevent a pathological situation from occurring.

In summary, as we have just seen GH plays a very important role in vascular homeostasis, acting on NO production, protecting from oxidative stress, regulating cell proliferation, differentiation and survival, regulating cell motility and migration, and inducing the release of EPCs and the differentiation of MSCs into vascular ECs. Since GH secretion decreases until it practically disappears as we age, it seems logical to assume that the loss of the hormone is one of the main causes of vascular problems that occur in old age.

3. Molecular aspects of GH/IGF-I in the vascular wall favoring arteriogenesis

3.1. GH/IGF-I response to shear stress forces (SSF): the mechanosensing pathway

In 2011 W. Schaper highlighted two key aspects of the arteriogenesis pathways: 1) only the inhibition of the whole production of NO could inhibit the collateral growth; 2) the mitogenic agent for the activation of smooth muscle cells (SMCs) from the vascular wall was still unknown. Although several candidates were given, mainly fibroblastic growth factor (FGF) and platelet derived growth factor (PDGF), none of them met all the characteristics required [83]. However, considering the key role of the GH/IGF-I axis in the vascular homeostasis, regulating eNOS and NO, as stated above, and since both GH and IGF-I are potent mitogens [84], even stimulated by PDFG, perhaps both hormones should be also considered as candidates for being that unknown mitogenic agent or agents, because there can be more than one. During this chapter we will try to demonstrate this statement.

Since the discovery of the shear stress forces (SSF) as the main stimulator of collateral enlargement during arteriogenesis, many investigations have been developed trying to find the connection between mechanical and biological aspects. Shear stress genes, activated by the stimulation of mechanical endothelial receptors, were proposed as sensitive elements for increased redirected flow through collaterals after arterial occlusion. However, some aspects have still to be elucidated, such as which molecules mediate the mechanical signaling pathway, or in other words, are there any factors involved in the arterial growth capable of being sensitive to mechanical forces? The answer to this question should be yes, and some of these factors could be locally produced hormones. Evidence accumulated in the last years has shown how the GH/IGF-I system can be regulated by multiple factors, such as growth factors, cytokines, lipoproteins, reactive oxygen species, and hormones or neurotrasmitters, but also, by hemodynamic forces [85]. As is known, many patients with arterial hypertension develop left ventricle hypertrophy and aortic wall thickening, while patients suffering an aorto-cava fistula develop hypertrophy of the right ventricle and an overload of the cava vein. Intuitively, one has to realize that something must orchestrate this adaptation to the pressure or the changes produced by volume. Given the strong mitogenic capacity of GH and IGF-I, and that they have many receptors in large vessels, such as the aorta or cava vein [84,86,87], there has

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to be a cross-talk between these hormones and hemodynamic forces. This hypothesis was demonstrated in an interesting study in which a volume or pressure overload was applied to rats to study the gene expression of GH receptors (GHR) and IGF-I mRNA in the vein cava and aorta. In the distended volume vein cava model, an 8-fold and 3.5-fold increase was found in IGF-I and mRNA, respectively, compared to control animals, as early as day 4. Besides, the IGF-I protein was located in SMCs. In the aortic stress model, a 4-fold and 5-fold increase in IGF-I and GHR mRNA, respectively, was found in pressured aortas at day 7. Both cava vein and aorta showed structural adaptations with a growth response. This study is very important, as it represents a possible way of connection between the mechanical and biological pathways, with a major role played by the autocrine/paracrine production of GH/IGF-I. The increase in vascular wall stress, therefore, seems to trigger overexpression of IGF-I and GHR mRNA in large vessels [87]. These data are consistent with the role of local IGF-I in blood vessel growth responses found in a rat model of aortic coarctation, with an increase of more than double IGF-I levels in the day 7, persistent on day 21, accompanied by a growth of SMCs and ECs, which agrees with the mentioned study [88]. Thus, in this model, IGF-I plays a pivotal role in the remodeling of the wall vessels in the aorta under high shear stress. Even more, the authors state that IGF-I mRNA levels are even high in quiescent aortic SMCs, which underlines the role of IGF-I as an autocrine growth factor for dynamic changes in the vascular wall [89]. An earlier report also showed the same data, but in a rat model of femoral artery overload, where strong immunoreactivity for IGF-I was detected in the middle layer of the left femoral artery 24 hours after the right femoral ligation, along with a significant decrease in the expression of IGF-I in the occluded artery in the zone distal to the occlusion [90]. All these findings provide evidence for a major role of autocrine/paracrine GH/IGF-I system in mediating vessel wall growth during shear stress (Figure 3).

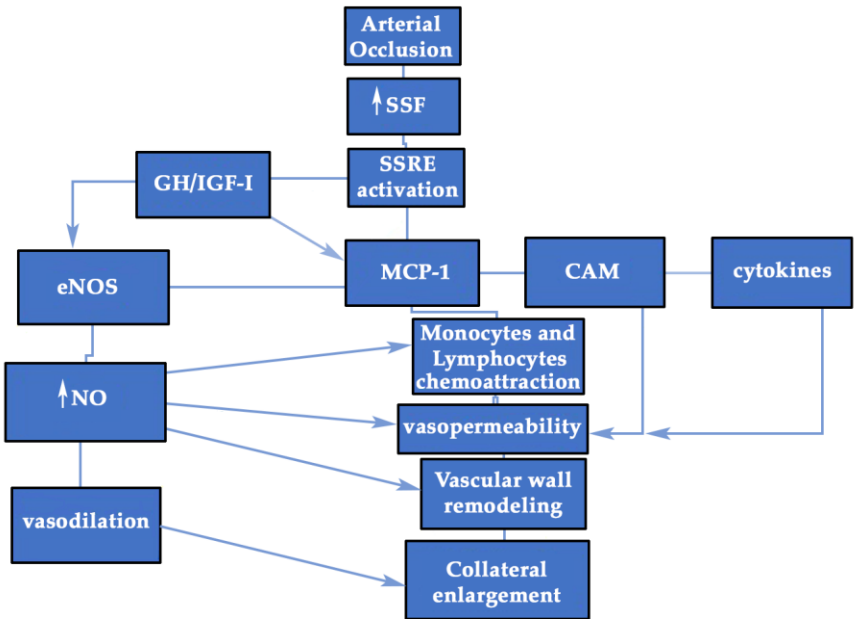

Figure 3. GH/IGF-I are also produced in response to shear stress forces (SSF) for collateral enlargement. Schematic representation of the activation of several molecules after the increase in SSF in which GH and IGF-1 have been added as a new element that enhance all these mechanisms. Since GH and IGF-1 are potent mitogenic agents, they have to play a role in the translation of mechanosensing signals. The NO pathway not only is important for vasodilation, but also has many actions in chemoattraction of inflammatory cells and vasopermeability. Local production of cytokines and hormones seems to be essential for collateral enlargement. SSF: shear stress forces; SSRE: shear stress response elements; eNOS: endothelial nitric oxide synthase; MCP-1: monocyte chemoattractant protein-1; CAM: cellular adhesion molecules; NO: nitric oxide.

For carrying out its mitogenic function, GH has been shown to directly stimulate Src family kinases (SFK), that in turn activates mitogen-activated protein kinase 1 (also known as extracellular signal- regulated kinase 2 (ERK2) and mitogen-activated protein kinase 3 (also known as ERK1)

through a phospholipase Cγ–Ras pathway [91]. The prolactin (PRL) receptor similarly activates the same SFK signals [84], and it is well known that GH interacts with the PRL receptor with the same affinity that it does with its GHR [92].

All these adaptations in the vascular wall by GH/IGF-I may be supported by the fact that GH is capable of modifying the aortic content and composition of collagen and elastin, and even its mechanical behavior [93]. With aging, a decrease in collagen and elastin in the aorta can be detected in rats and humans parallel to the GH/IGF-I decline, although plasma IGF-I is still maintained at lower levels, since its hepatic production does not depend exclusively on GH, which practically disappears in the elderly [1]. In fact, the administration of GH to female old rats augmented the collagen deposit in a 300% and its turn-over in the media layer. It seems that the main mediator of this GH effect in the aorta is IGF-I, produced locally in SMCs. In the thoracic of old female GH increased the ration of collagen I/III, improving both the stiffness, in those areas under high overload, and the extensibility in those with low overload, thus adjusting the aortic mechanical characteristics to the overload [93]. These studies shed some light on the idea that local production of hormones takes part in the regulation of structural adaptations of blood vessel under stress conditions; for it, the number of IGF-IR plays a major role for the SMCs response. On the one hand, the increase in the expression of IGF-IR by GH or other factors such as angiotensin II or FGF-2 is crucial for the mitogenic effects of IGF-I on SMCs. On the other hand, factors such as TNF-α or ox-LDLc decrease IGF-IR, favoring SMCs apoptosis. Effectively, GH and IGF-I play a key role in vessels for the growth and survival of SMCs, inhibiting apoptosis produced by mitochondrial dysfunction induced by TNF-α and ox-LDLc [85].

3.2. GH and GHR: a complex regulation explaining different results.

The vascular wall is a special target for GH, since many GHR have been described in the vascular system depending of the vascular bed, and it is clear that many of the effects of GH on the vascular wall are independent of IGF-I [7,92,94,95]. Indeed, even though the GH/IGF-I axis is highly coordinated to act, both GH and IGF-I have independent actions [96]. For example, the insensibility to GH in Laron syndrome is not fully corrected with IGF-I administration [84]. Moreover, the endocrine or local production of GH/IGF-I exerts different actions. For example, pituitary GH is secreted in pulses, some of them of great amplitude, while autocrine GH is produced almost continuously and at low levels; these differences lead to the different actions that both types of GH have. Maybe this is the reason why the oncogenic potential seems to be reserved only for local GH [11], and not for the pituitary or exogenously administered hormone. Moreover, both type of GH production regulate gene expression in a different way.

In the vascular wall, although GHR have been found in different arterial layers, the endothelium appears to be it is found in great amounts. In the media layer many of the GH actions are mediated by IGF-I, especially in SMCs [93], or they occur as a consequence of diffusing signals generated by GH in the endothelial layer, as diffusing NO. With independence of the cardiovascular system, one of the most interesting location of GHR is in the endothelium of vessels of endometrium, where GH seems to have an important action in the creation and maintenance of vessels during every sexual cycle in females [97]. This fact is extraordinarily important, since it represents one of the key aspect that supports the role of GH in arteriogenesis, mimicking the physiologic process that takes place in fertile women [79].

As widely described, GH induces eNOS expression and NO release in cultured human ECs [94]. When L-nitroarginine methyl ester (L-NAME) is administered, GH loses its action on NO, which confirms this main action on the endothelium [98]. To produce NO, eNOS has to be phosphorylated on serine 1177 via PI3K/Akt, activated by a calcium-calmodulin [99]. Diffusing NO triggers a soluble guanylyl cyclase into SMCs, increasing intracellular levels of cyclic GMP (cGMP) that activates protein kinase G1 via phosphorylation of the inositol-triphosphate receptor-associated cGMP kinase and the sarcoplasmic reticulum ATPase. The consequence is that intracellular calcium decreases provoking vascular relaxation and modifying the arterial tone [99,100], one of the most important action of NO and GH, demonstrated after arterial infusion of the hormone [101]. It has to be underlined that eNOS activity is highly regulated in cells at transcriptional level and by other factors

such as acylation and phosphorylation, or by protein-protein interactions [99]. However, this is not the only benefit of NO on the vascular wall, as it facilitates vasopermeability (by decreasing VE-Cadherin activity), monocytes chemoattraction, reduces lipoxygenase activity and ox-LDLc, platelet adhesion and SMCs proliferation and migration [13,102]. As described before, this is the main pathway utilized by GH to contribute to vascular homeostasis. However, when a pathological condition is present as ischemia, GH effects can change, as they adapt to the environmental circumstances.

High GHR expression has been detected in the aorta, femoral and carotid arteries, where it exerts many effects induced by GH directly by activating the JAK/STAT pathway, among others [84,86], as GH is unable to induce IGF-I transcription in ECs [92]. This does not means that GH does not interact with IGF-I in the media layer where precisely regulates growth and survival of SMCs mediated by IGF-I, for which SMCs have many receptors [85]. GHR is crucial for the wide effects of GH, allowing the hormone to internalize in the cells where GH triggers many signaling pathways and can influence gene transcription. This internalization is facilitated by GH-binding proteins (GHBP), the extracellular component of GHR. The concentration of GHR in a determined tissue determines the intensity of GH signals, mainly when considering the eNOS-NO pathway. The higher the number of GHR, the greater the effect of GH, but up to a limit where if we continue to administer more hormone, the effect not only does not increase but decreases. For instance, 1 nmol/L of GH produced 6.39 µmol/L NO; 10 nmol/L of GH, 6.45 µmol/L NO, but 100 nmol/L of GH produced 6.38 µmol/L NO [103]. It is important to point out that the own GH takes the control of the gene involved in the expression of its receptor, and that this regulation depends on the time and dose of hormone administration. When a physiological dose is given, GHR is upregulated increasing until 48 hours after the administration of GH. In situations where supra or infraphysiologic doses are used, the behavior of GHR is different, usually undergoing a downregulation [103,104]. This fact is important to understand the reasons why GH has different effects depending on the dose, and to understand why clinical trials with GH sometimes differ in their results. As explained before, the relationship between GHR and GH is bidirectional, and GHBP binds to the free hormone in the blood to control the amount of GH that interacts with its receptor, but also to regulate the GH clearance. Even inside the cells, GH actions are exhaustively regulated, since when the control is loosed, severe consequences can appear. This is an important concept for all the growth factors acting in the organism. The effects of GH on GHR gene expression seem to be dependent on the time/dose of exposure in addition to the cell type and if the experiment is carried out *in vivo* or *in vitro*. But GHR gene expression is even more complex, as it is also influenced by other factors as nutritional intake, medications such as steroids or morbid conditions such as diabetes mellitus. Furthermore, the GHR gene has multiple 5' untranslated exons controlled by multiple promoters, depicting the complexity and high quality regulation of the gene expression of this receptor [105].

3.3. GH/IGF-I can favor inflammation during collateral enlargement

During collateral enlargement two cells will be activated to achieve the great change, both endothelial and SMCs will acquire a proliferative and secretory phenotype after a previous phase of NO-dependent vasodilation, because hemodynamic or short-term compensation always runs first than that which originates from growth factors or long-term compensation. While in physiological situations the flow and metabolism control is done mainly at local level, when a pathological condition as ischemia is present the systemic response is usually required, focused on flow redistribution and microvascular adaptations [106], which will need of the neurohormonal axis effects. In both circumstances, local and systemic GH/IGF-I will be important to regulate short and long-term adaptations.

The inflammation of the collateral wall aims to fill the same of cells that will accomplish the vascular remodeling. To do this, two stages can be distinguished: first, ECs will facilitate the inflammatory response in the vascular wall; second, SMCs will proliferate to increase vessel size. It is not the aim of this chapter to describe the whole process of arteriogenesis, but to highlight those aspects in which GH could participate. For example, it has been described how monocytes, monocyte chemoattractant protein-1 (MCP-1), and lymphocytes are crucial for vascular remodeling during

arteriogenesis during the inflammatory phase [107], since this process is not complete or is delayed when it lacks of all these molecules. It has been shown that GH strongly induces these cells and proteins [1,108,109], playing a pivotal role in the chemotaxis and migration of human monocytes.

MCP-1 has been found elevated in blood samples from the collateral network of coronary arteries [110], and transiently and selectively increased in the ischemic muscle during the first 3 days after ischemia [111] underlining its importance in arteriogenesis. The relation of GH and MCP-1 has been conveniently studied and seems to be mediated via Janus kinase 2 (JAK2) and p44/42 mitogen-activated protein kinase (MAPK) activation, since MCP-1 significantly decreased in cells pretreated with the JAK2 inhibitor AG490 or MAPK inhibitor PD98050 [109]. In the aforementioned experimental study, MCP-1 mRNA levels augmented up to 8 times after low dose administration of GH [109]. As is known, both MCP-1, for the attraction of inflammatory cells, and cell adhesion molecules (CAMs), for the adhesion and invasion of the vascular wall for this type of cells, play a central role in igniting the arteriogenic process. But CAMs are also related to GH, especially vascular cell adhesion molecule-1 (VCAM-1). GH significantly increases the expression of VCAM, as demonstrated when the serum of healthy patients treated with the hormone is administered to cultured umbilical vein ECs [112]. It has been proposed an indirect mechanism for this action, maybe mediated by VEGF, IGF-I or SDF-1 [16,92,106,113], that upregulate CAMs [107]. This action is also seen in GHD adults, in which GH replacement therapy significantly increases VCAM-1. Therefore, both MCP-1 and VCAM could potentially be modulated by GH, facilitating the first phase of arteriogenesis. In addition, the CD34 antigen from CD34 + cells is considered as an adhesion molecule that is though that regulates cell migration and cell adhesion mediated by integrins [114]. GH potentiates CD34+ cells and their function, supported by the fact that circulating CD34+ are decreased in adult GHD which contributes to endothelial dysfunction, and GH replacement therapy increases the number of these cells improving endothelial function [115], necessary for arteriogenesis.

Almost all human immune cells (B lymphocytes, T lymphocytes, natural killer cells and monocytes) in human blood express GHR, and produce its ligand that may act in an autocrine or paracrine manner. GHR has been described in 30 % of human peripheral blood mononuclear cells (PBMC) through a binding study [116,117], and human GHR was detected in more than 90% of B lymphocytes and monocytes, with a lower expression rate in T lymphocytes [117]. Evidence suggests that B lymphocytes are the main producer of GH. Human immune cells mainly express the hGH-N gene that encodes the molecular forms of 22 KDa and 20 kDa, the same GH found in the anterior pituitary gland [118]. The immune system also has receptors for PRL, which are activated by GH as stated above [117]. In addition, immune cells have IGF-IR and produce IGF-I. This could be another indirect way to influence these cells by GH [119,120]. All the mentioned cells participate during the vascular wall inflammation needed for collateral enlargement. These findings suggest that immune cells respond to GH with their activation. Thus, first, GH helps immune cells to migrate and to invade the vascular wall, and second, once in the vascular wall, GH activates them directly or indirectly to produce cytokines (Figure 4).

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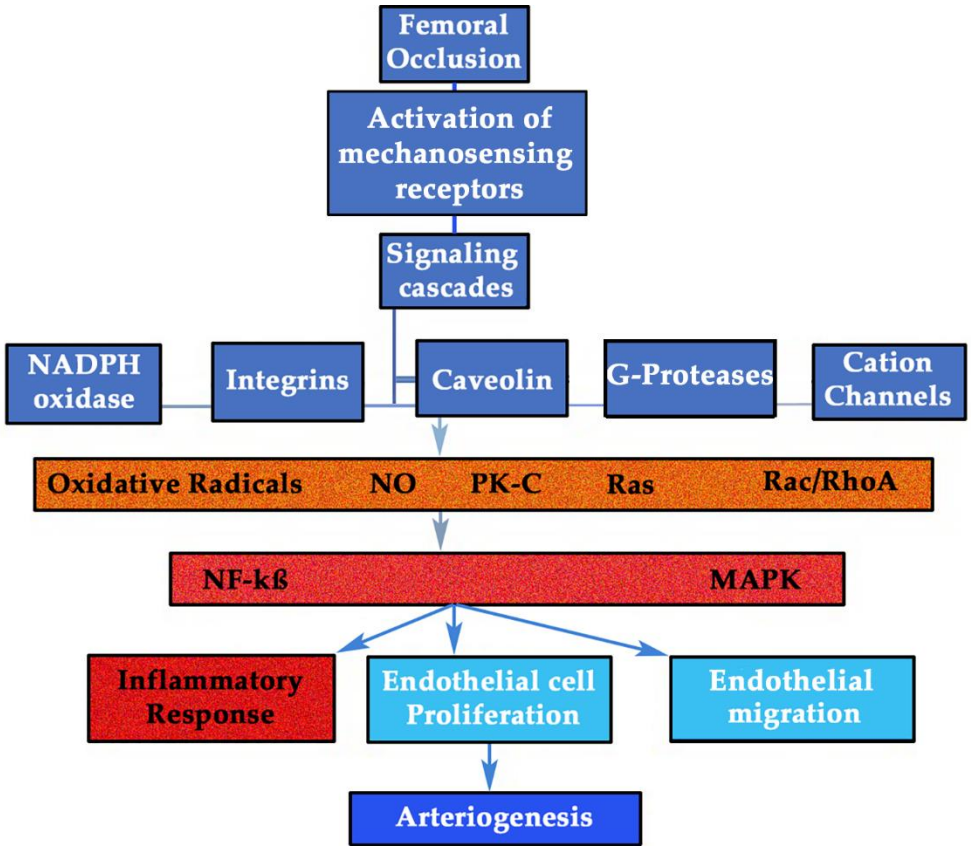

Figure 4. Signaling cascades for collateral growth: redox system can play a major role.

Schematic representation of the signaling cascades after an arterial occlusion, triggering both the inflammatory response and endothelial cells proliferation and migration phenomenon into the media layer. Several pathways have been highlighted: Rho, involved in endothelial cells proliferation; Ras, for endothelial cells migration; NO, for endothelial function and monocytes adhesion. Oxidative radicals from cell metabolism are currently considered very important in the stimulation of the NO pathway for vascular homeostasis. They control NO bioavailavility. Rho pathway has been advocated as crucial for SSF sensing. Caveolins: family of integral membrane proteins that play a role in the integrin signaling and in migration of endothelial cells. Cation channels, mainly ion Ca^{2+}, are also related to PKC and the RAS/RAC activation. All signaling cascades are activated by shear stress forces. For more details see reference [121,122]. NADPH oxidase: nicotinamide adenine dinucleotide phosphate oxidase; NO: Nitric Oxide; PK-C: Protein kinase C, Ras: rats' sarcoma-extracellular signal-regulated kinases; Rho, hexameric protein found in prokaryotes, necessary for the process of terminating the transcription of some genes, Rac: Ras-related C3 botulinum toxin substrate (subfamily of the Rho family); NF-kß: nuclear factor-kappa ß, MAPK: mitogen-activated protein kinase.

It is noteworthy that monocytes have to be activated to help in arteriogenesis, since when they are transplanted directly from the blood in animal models of ischemia they do not influence this process. However when they are previously activated by the monocyte colony stimulating factor (M-CSF), collateral growth begins [123]. Thus, M-CSF favors the suitable environment for a stable monocyte function [18]. This is a very important finding, especially when GH is strongly related to M-CSF. Recombinant human GH (rhGH) stimulates chemoattraction of human monocytes and random migration at picomolar concentrations of the hormone in both *in vitro* and *in vivo* studies.

These effects are parallel to plasma GH levels. GH-activated monocytes release superoxide anion and cytokines without changes in plasma levels of TNF-α or IL-6, which discards that this effect was mediated by cytokines secreted by monocytes [124]. The cellular pathway involved was demonstrated many years ago [125], as GH stimulates the tyrosine phosphorylation of two proteins p130Cas and CrkII, their association and the association of other multiple tyrosine-phosphorylated proteins that end up activating the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) [126]. Such a large multiprotein signaling complex is not only the basis for activating monocytes, but it is also essential for other effects such as cytoskeletal reorganization, cell migration, chemotaxis, mitogenesis, and/or the prevention of apoptosis and gene transcription [125].

Monocytes have traditionally been reduced to immune actions, but they can also have trophic effects in homeostasis, for instance secreting CSF during growth. In fact, when CSF-1 lacks many skeletal defects or deformities have been described in animals along with a delayed growth, while the administration of recombinant human CSF-1 corrects these changes [127], suggesting that this factor interacts with the GH/IGF-I axis. Macrophages also produce IGF-I in response to CSF-1 and other stimuli [120,128], and represent one of the main producers of extrahepatic IGF-I [129]. Although at a lower level, other types of the M-CSF family, such as G-CSF or GM-CSF, have the same effect on the production of macrophage IGF-I. Furthermore, IGF-IR is highly expressed in macrophages stimulated by CSF-1. However, the specific link between CSF-1 and GH has not yet been adequately studied, although it has been seen that many CSF-1-dependent macrophages appear in the pituitary gland in the course of somatotropic cell development [130]. Together with IGF-I, FGF-2 is also produced by activated monocytes, which participates in the proliferation of SMCs. While GH does not increase endothelial IGF-I, this hormone may have a higher impact on the production of IGF-I or FGF-2 at the media layer level.

3.4. GH/IGF-I and eNOS in arteriogenesis. Insights from GHAS trial: is redox balance the main actor?

As we have just seen, several signaling pathways work together to grow the collateral arteries, and one of the most relevant, although with some contradictory results, is the NO pathway. This pathway has been considered during many years crucial for collateral enlargement, both during the early and late stages of arteriogenesis. Measuring several variables such as perfusion of the limb, the diameter of the collateral artery and the number and location of pericytes within the ischemic hindlimb, less recovery of blood flow and a smaller collateral artery diameter have been demonstrated in the group of eNOS-/- and L-NAME mice than in the wild-type. The pericytes barely appeared and they did so in a random pattern in the former groups compared to the wild-type [131]. Another fact that supports the role of this pathway in arteriogenesis is the finding of a lower eNOS expression both in the thigh and in the gastrocnemius muscles in diabetic mice, which reduces collateral enlargement. The reduced expression of eNOS in diabetic mice (types 1 or 2) may contribute to the deficient arteriogenesis and angiogenesis seen in these animals, while the treatment with thiazolidinediones, that can increase eNOS activity through the peroxisome proliferator-activated receptor (PPAR) gamma [132], could restore these deficient responses [131]. Even more, animal models of ischemia after training support the important role of eNOS and NO in arteriogenesis, with an even greater effect than that induced by VEGF, because the latter is a secondary factor for collateral growth and eNOS a primary factor, unlike what happens in angiogenesis, where the role of eNOS is less relevant, but VEGF also needs it for its actions

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[133]. Therefore, the difference in NO dependence is the key factor that distinguishes between both processes.

However, not all authors agree completely, since it seems that eNOS and NO could be of great importance for NO-mediated vasodilation of peripheral collateral vessels after arterial occlusion, but their relevance seems to be minor for collateral enlargement. Tissue perfusion and collateral-dependent blood flow increased significantly in mice overexpressing eNOS compared to the wild-type, but only immediately after ligation. In eNOS-/- mice, collateral-dependent blood flow kept poor until day 7, and after that it recovered, suggesting only a delay, but not a complete impairment of collateral growth. Besides, no differences in collateral arteries between the three groups of mice were histologically confirmed at the end of the study, and the administration of an NO donor induced vasodilation in collateral arteries of eNOS-/- mice, but not in the wild-type [134]. Interestingly, eNOS deficiency may be compensated by iNOS activity [83], and that might be the explanation of this finding and other different results. That is, the exact role of eNOS is not fully understood. Nevertheless, some insights from the GHAS trial, conducted by our group could be helpful in clarifying the activity of eNOS in humans. In this randomized controlled trial, GH or placebo was administered to patients with critical limb ischemia without revascularization options, and ischemic samples from calf muscles were taken at baseline and after 8 weeks of treatment. The data obtained showed us a surprising finding: in the group treated with GH (0.4mg/day for 8 weeks), there was an increase in the level of eNOS mRNA compared to baseline, but this increase was significantly lower than that detected in control patients treated with placebo, and parallelly, a significant and strong decrease in NOX4 levels was only observed in the GH group (Figure 5); this means that the activity of eNOS depends largely on the redox balance in the ischemic muscle or even more, that the arteriogenic growth of the vessels depends largely on the redox imbalance and that GH, by correcting this stress, also reduces the signal that stimulates eNOS.

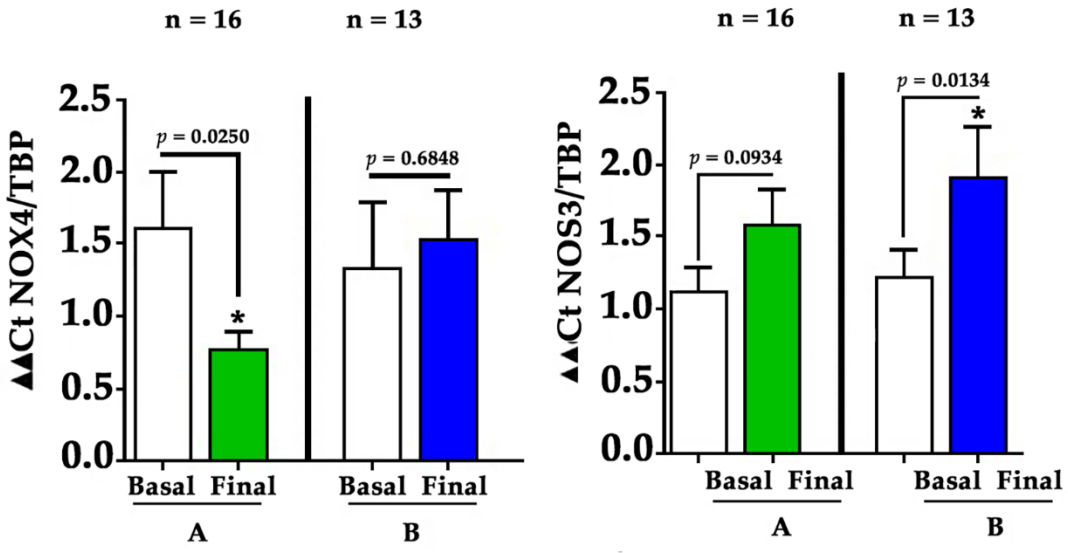

Figure 5. GH decreases the redox stress in ischemic muscles of patients with critical limb ischemia. Insights from the GHAS trial.

Left graph shows the significant decrease in NOX4 mRNA levels only seen in GH group. Right graph depicts the significant increase in the levels of NOS3 (eNOS) mRNA levels during the period of treatment (8 weeks) in patients with placebo related to the baseline levels, while in GH group, although there was

also an increase in NOS3 mRNA, this was not significant. Muscle samples: soleus muscle from ischemic lower limbs. Group A: GH; Group B: placebo. Statistics: Non-parametric test. Normality test: Kolmogorov-Smirnov.

This is an important concept because it indicates that there is an insensibility to NO rather than a real depletion of the same or eNOS in patients with peripheral ischemia [135], and this finding matches perfectly with elevated redox stress and insensitivity to NO found in humans and animals with peripheral arterial disease by other authors [136–138]. This is also consistent with the fact that an increase oxidation can be seen in GHD-adults, secondary to an elevated activity of lipoxygenase activity and ox-LDLc, main source of atherosclerotic lesion of the arteries, as a consequence of the NO depletion. After GH replacement therapy, this redox alteration can be significantly reduced, together with the recovery of a normal flow mediated dilation test, compared to pre-GH administration and control patients [13]. At this point, it is of interest to remark that senescence, frequent in patients with peripheral artery disease, is like a GHD state.

Therefore, the important message here is the relevance that redox balance exerts on collateral enlargement. As it has been described, while small or acute redox stress, characterized by an elevated reactive oxygen species (ROS)/reactive nitrogen species (RNS) production, seems to influence positively on neovascularization, a chronically high level of oxidation will be detrimental for vascular growth and remodeling [139]. In the GHAS trial, patients under placebo treatment maintained a high level of NOX4 and eNOS mRNA in the calf muscle, trying to compensate for the decrease in NO bioavailability, but those under GH treatment, stopped this vicious circus, lowering the redox imbalance, and also increasing NO bioavailability without a necessity of an increase in eNOS production. Effectively, the bioavailability of NO depends to a large extent on the production of ROS, since they will react with NO to inactivate it, lowering their levels [140], which means that the downregulation of the redox stress by GH in patients with critical limb ischemia upregulates NO bioavailability with a no significant elevation of the eNOS levels in their ischemic muscle. This unexpected finding highlights the dynamic role of redox balance in vascular homeostasis. Typically NOX4 is upregulated in cells by SSF, hypoxemia or cytokines like TNF-α, and the latter is usually elevated in patients with ischemic process [141], favoring inflammation and phosphorylation of eNOS, which reduces NO production [142]. In the GHAS trial, GH also significantly reduced circulating levels of TNF-α, which is consistent with other studies [143,144], and with the fact that improves redox balance, representing another way to act in this process (Table 1). TNF-α has other negative actions, elevating intracellular SOCS [106], or diminishing IGF-I or SMCs in the vascular wall [85]. GH, as seen, could correct these negative effects, decreasing TNF-α levels. Although a proinflammatory activity has also been seen for GH, this is something that occurs when high levels of the hormone are considered, demonstrating, once again, that the role of GH depends on its physiologic or pathologic concentrations and on the time of instauration of the morbid condition (acute or chronic) [145].

	Group A			**Group B**			
	Obs.	Mean	SD	Obs.	Mean	SD	*p* Value
TNF-α 0	16	12.35375	5.20452	16	8.7875	3.9441	**0.0184**
TNF-α 2	15	10.928	5.128023	14	8.041429	3.600089	**0.0464**

Table 1. GH decreases inflammation by lowering circulating levels of TNF-α in patients with critical limb ischemia. Insights from the GHAS trial.

TNF-α levels (TNF-α 0) were significantly elevated in the plasma of patients treated with GH (group A) compared to placebo (group B) at baseline, which means that the ischemic process was more severe in group treated with the hormone. However, after two months (8 weeks) of treatment, only patients under GH treatment

showed a significant decrease of circulating levels of TNF-α (TNF-α 2). Determination of TNF-α levels in plasma: ELISA test (Quantikine, R&D Systems). Normal reference value for TNF-α levels: < 8.1 pc/ml.

Regarding to the ischemic disease, authors sometime do not agree because they are not describing the same phenomenon, since an acute injury is normally produced in the animal model of ischemia, while a chronic process is usually developed in the human being with peripheral or coronary artery disease. In fact, in human heart, after acute coronary occlusion the arteriogenic phenomenon is very fast, as in animal models, between 1 to 2 weeks for the great majority of patients [146], while in chronic ischemia the collateral enlargement takes several months [147]. This is a concept that needs to be highlighted, as in the chronic ischemic state, due to the lower and slower activation of the physiologic compensation, impaired by age, GH could be of help, powering all these mechanisms and gaining a high distal flow.

When both acute and chronic animal models of ischemia are used simultaneously, it was shown that the mechanisms regulating blood flow recovery, gene expression, macrophage infiltration and recruitment of hemangiocytes are critically different, since they are dependent on the arterial occlusion rate, and the mechanisms regulating blood flow recovery also differ [148]. The most important finding in the former study is that MCP-1 or shear stress-induced genes as eNOS or Egr-1, are not sufficiently activated to induce collateral artery enlargement in the model of gradual ischemia, since SSF through collaterals are weaker compared to those in the acute ischemia model, and eNOS or MCP-1 are less stimulated in gradual or chronic ischemia than in the acute process at the level of the thigh, place where collateral growth mainly takes place collateral growth. Although upregulation of VEGF and PlGF is found in acute models, particularly important are eNOS and KDR/Flk-1 in the remodeling of vessels [133], while in humans, as stated above, it seems that lowering the redox imbalance could be more profitable. Interestingly, in the GHAS trial we also found a parallel and significant increase of KDR/flk-1 mRNA levels from muscle samples in the GH group in the GHAS trial (Figure 6A), what confirms VEGF and, probably, EPCs activation, a finding consistent with that from animal models of hindlimb ischemia treated with IGF-I plasmid [149]. This effect could be dependent of IGF-I, but we hypothesize that it is a direct action of GH on muscle, because a parallel increase of IGF-I mRNA has not been found, at least at the time in which muscle samples were obtained (Figure 6B). However, the elevation in KDR/flk-1 was not accompanied by a parallel significant increase of VEGF mRNA levels after two months of treatment (Figure 6C). This fact has an explanation. Knowing that VEGF mRNA levels decrease after stimulation with very low levels within the 4th week [150,151], it is easy to understand that, simply, we got the extraction of the muscle sample when the levels of this factor had already declined (at 8 weeks), overlooking the possible elevation produced during the previous month.

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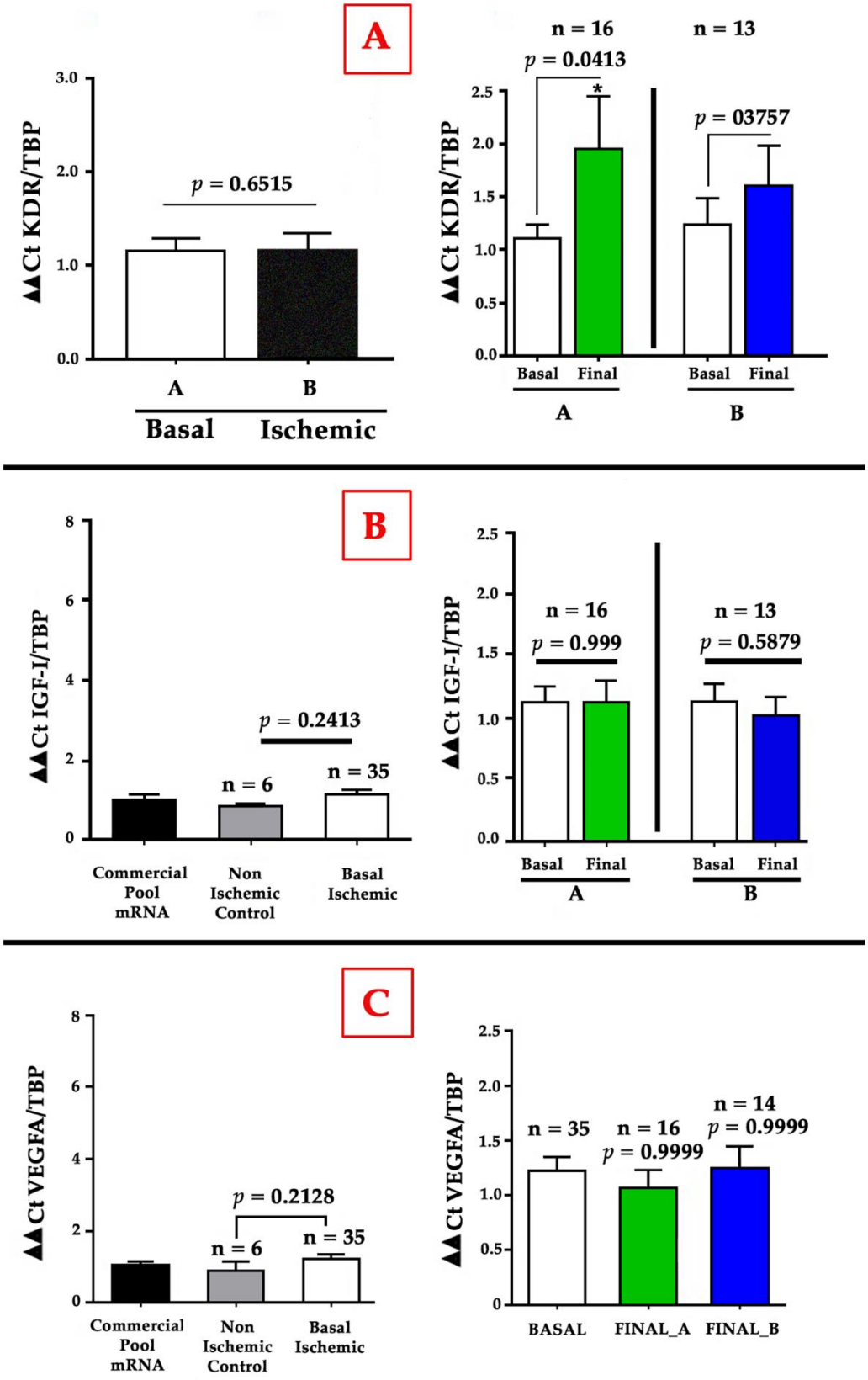

Figure 6. GH increases VEFG-R2/Flk-1(KDR) mRNA levels in ischemic muscles of patients with critical limb ischemia without apparent changes in IGF-1 and VEGFA mRNA. Insights from the GHAS trial. **(A).** VEFG-R2/Flk-1(KDR) mRNA levels in the GHAS trial. Left graph shows baseline levels between both groups of treatment with no significant differences. In the right graph, a significant increase in KDR mRNA levels in GH group related to placebo during the period of treatment (8 weeks) can be found. **(B).** IGF-1 mRNA levels in the GHAS trial. Left graph shows levels of IGF-1 at baseline in ischemic vs non-ischemic muscle samples with no differences. Right graph shows the lack of changes in any group of treatment during the period of treatment. **(C).** VEGFA mRNA levels in the GHAS trial. Left graph depicts the comparation between VEGFA levels in ischemic vs non-ischemic muscle samples in the calf at baseline from the GHAS trial. Right graph depicts the lack of significant changes after 8 weeks of treatment. Group A: GH; Group B: placebo. Non-ischemic muscle samples: sample of reference obtained from amputations in patients without limb ischemia. Commercial pool mRNA: is a commercial RNA from skeletal muscle used as a technical control for normalization in trials. A non-parametric test was used for the statistics. Normality test: Kolmogorov-Smirnov.

This finding is also very important, as it has been advocated that the differences between VEGF action in different tissues might be determined by the number of its receptors rather than by the levels of VEGF [152], that usually are normal in patients with peripheral arterial disease at the calf [153], albeit with controversial results, as while in acute ischemia VEGF and VEGFR-2 are diffusely expressed in the affected muscle, in skeletal muscle that recovers from chronic ischemia the former factors are restricted to atrophic and regenerating muscle areas [154]. This is consistent with the fact that KDR/Flk-1 expression was revealed in macrophages and fibroblast cells of the necrotic area after myocardial infarction [155]. Here we present a clear evidence that VEGF is not normally augmented in distal limb muscles under chronic ischemia as compared to control muscle samples obtained from limb amputations in non-ischemic patients, and that GH can increase VEGF actions through, at least, a raise of the KDR receptor, also favoring muscle regeneration that has to be accompanied by vascular development. As known, KDR mediates most of VEGF actions on mitogenesis, survival and permeability at the vascular wall, being mainly expressed in ECs but also in macrophages and SMCs. However this receptor has been found in myocytes from skeletal muscles both in the membrane and in the cytoplasm [154], and then KDR could be derived from myocytes and, from this point of view, GH could be acting more at the muscular level rather than at the vascular one. The immunohistochemical analysis of the study samples will have the final answer when it is carried out.

In any case, as is known, the increase in SSF after an arterial occlusion triggers the NO pathway, which inhibits the expression of VE-cadherin, responsible for maintaining the vascular membrane integrity and, therefore, increasing the vascular permeability and the invasion by inflammatory cells of the vascular wall [156]. Both disruption of endothelial junctions and remodeling of the cytoskeleton are necessary for vascular permeability, and NO released by the endothelium is crucial. One approximation of the molecular mechanism involved has been shown in a study in which the lack of eNOS reduces VEGF-induced permeability mediated by an increase of the Rac GTPase activation. The depletion of NO impaired the recruitment of the guanine-nucleotide-exchange factor (GEF) TIAM1 to adherent junctions and VE-cadherin, and reduced Rho activation. NO is crucial for Rho GTPase-dependent regulation of cytoskeleton architecture leading to reversible changes in vascular permeability. It seems to be clear that when NO is inhibited, flow-induced arteriogenesis is also interrupted [133].

3.5. GH and CXCL12 (SDF-1): a potential collaboration for collateral growth

Another cytokine that plays an important role in arteriogenesis is SDF-1. This cytokine, produced mainly in platelet or EPCs, can help collateral growth by stimulating the recruitment of hemangiocytes recruitment, that will be integrated into the wall of vessels as collaterals. This phenomenon is inflammation-dependent: the greater is the inflammation, the greater the recruitment of these cells, something that usually occurs in acute ischemia. Maybe this explains why SDF-1 plays a lower than expected role in gradual ischemia. Nevertheless, SDF-1 has two important actions in this last process: one, the aforementioned recruiting of hemangiocytes that will differentiate in SMCs in the arterial wall, collaborating in collateral enlargement; two, the recruitment of EPCs and smooth muscle progenitor cells in skeletal muscle.

Particularly striking is the relationship between SDF-1 and GH, since CXCR4 has been found in pituitary somatotrophs, where SDF-1 activates the expression of the GH gene and the production and secretion of GH from the anterior pituitary, regulating the normal physiological function of GH cells. In fact, when SDF-1β was administered to rats, both alone or with GHRH, the production of GH from the pituitary gland was increased in 2.5-3.5 times in a dose-dependent manner [157]. Interestingly, despite the different types of cells in the anterior pituitary gland, only somatotrophs express CXCR4. That means that SDF-1 and GH are closely related, at least at the pituitary level. The cellular mechanisms of this stimulation have been studied in GH4C1 cells, showing two possible pathways: Ca^{++}-independent stimulation of ERK1/2 activity and Ca^{++}-dependent activation of Pyk2 and BK_{Ca} [158]. This positive relation is also supported by the finding that GH stimulates SDF-1, since high levels of the same can be seen in the thymus of GH-transgenic mice and in cultured primary thymic epithelial cells derived from these animals, compared to age-matched wild-type counterparts. Besides, thymocytes migration induced by SDF-1 is improved when these cells are exposed to GH [159]. Since both SDF-1 and GH are coordinated during embryological development for vasculogenesis and for immune system function [16,159], it is tempting to speculate that both may act together in situations such as ischemic injury, mainly recruiting EPCs from the bone marrow and stimulating and leading the migration of macrophages and lymphocytes to the vessels.

3.6. GH, Progenitor cells and Thymosin B4: just another tempting speculation?

3.6.1. GH and endothelial progenitor cells (EPCs)

The discovery of EPCs mobilization in the bloodstream with the administration of low doses of GH in healthy subject is somewhat notable [55,160], since they play an essential role for angio- and arteriogenesis, especially secreting growth factors [161]. Although in clinical trials the injection of EPCs seems to be more effective for angiogenesis than for arteriogenesis, they can definitely contribute to collateral enlargement without any doubt, as they are crucial during vasculogenesis, a process in the embryo similar to the arteriogenic process in the adult. Indeed, a stimulation of angiogenesis and arteriogenesis was shown after treatment with wild-type EPCs, effect blunted when thymosin β4 (Tβ4) knock down EPCs were used, affecting both collaterals and microcirculatory vessels [161–163]. Tβ4 seems to require the PI3K/AKT pathway signaling for the induction of neovascularization, as it has been demonstrated in peripheral ischemic disease [164]. GH undergoes a deterioration parallel to EPCs with aging, with a corresponding decrease in klotho or GHRH, and a decrease in cellular response to GH [165]. As stated, the administration of GH not only improves the number of EPCs, but also restores the loss of their function while aging. This is interesting, because maybe the connection between EPCs and GH could come from the hand of Tβ4. That is, when GH is administered to hypophysectomized rats Tβ4 increases its thymus concentration in a dose-dependent manner. In fact, both molecules seem to work together during sexual differentiation [166], and then, both have to be related. From the knowledge that GH typically stimulates Akt gene expression [151], this could be a way to maximize Tβ4 actions and EPCs function. The mechanism by which EPCs are stimulated by GH is not completely understood, but it does not seem to be due to a direct stimulation of the bone marrow. Perhaps this is a secondary action as a result of the release of other factors, such as VEGF, erythropoietin (EPO), SDF-1 [160], or even the same IGF-I, for which receptors have been detected in EPCs [55]. From the point of view that both the number of EPCs and their function are impaired in patients with peripheral arterial disease, especially in the elderly [167,168], GH could help to improve both characteristics that limit the possibility of EPCs to be incorporated into collaterals. Figure 7 depicts EPCs evolution in placebo or GH group in a small sample from the GHAS trial.

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	Baseline			Final		
	Number	% Viable EPC	EPC/μL	Number	% Viable EPC	EPC/μL
Mean B (n=3)	6	0.0004	0.1574	1	0.0001	0.1265
Mean A (n=2)	1	0.0001	0.1214	9.5	0.0006	0.2563
Healthy (n=2)	5.5					

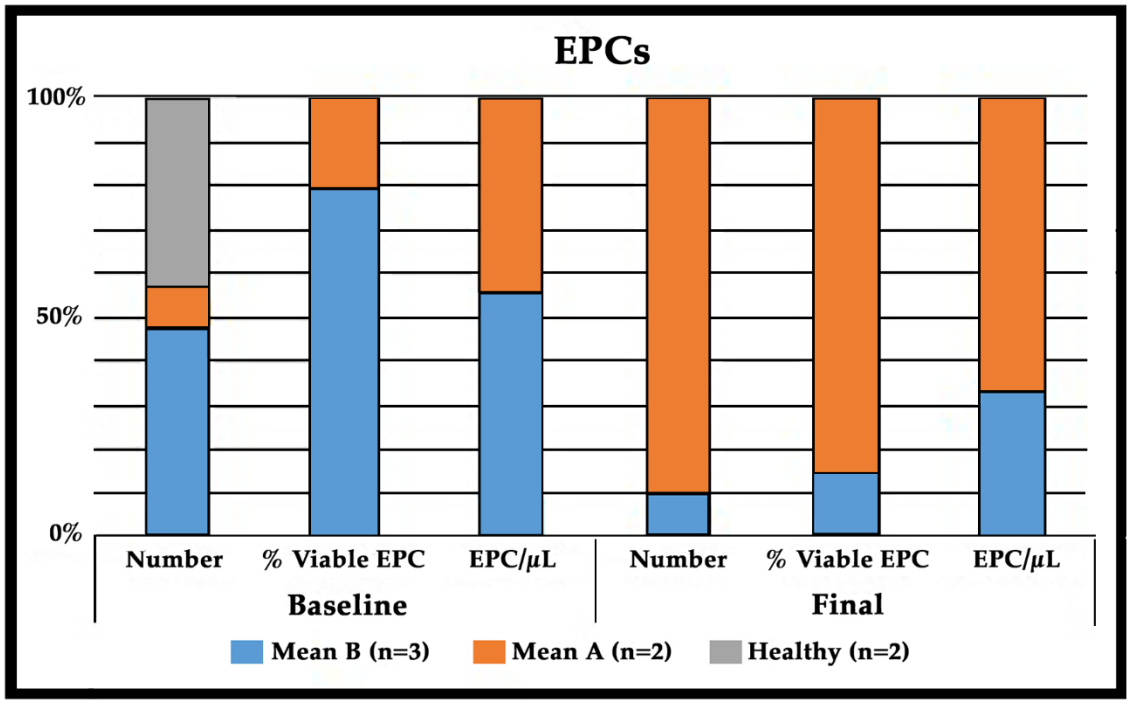

Figure 7. Circulating levels of Endothelial Progenitor Cells (EPCs) could be increased by GH administration in patients with critical limb ischemia. Insights from the GHAS trial.
This graph shows a comparation of the number of EPCs in five patients with critical limb ischemia and two healthy volunteers. While placebo group (B) seems to show a decreasing tendency during the period of treatment, conversely GH group seems to show a tendency to increase. This means support the published positive effect of GH on EPCs. GH group had lower number of EPCs at baseline, probably traducing a higher severity of ischemia, but EPCs increased after 8 weeks of GH administration. This is also consistent with table 1, in which biomarkers also show the same phenomenon: a higher level of ischemia in GH group patients at baseline. As a consequence of the small number of patients, no strong conclusions can be drawn.

3.6.2. GH and mesenchymal stem cells (MSCs)

Another intriguing relationship of GH is that the hormone has with mesenchymal stem cells (MSCs). These cells migrate in the embryo to the newly formed vessels to release growth factors and stabilize the new vascular network, since they can differentiate into pericytes. But these cells do not disappear in the postnatal period, and can be identified in adults in many tissues, especially in the bone marrow, but also in adipocyte tissue or in muscles near vascular structures. In adults, MSCs are also called resident stem cells, and constitute a reserve to replace damaged cells, since they are capable of differentiating into a wide variety of cellular types, reason why they have been advocated in tissue-regenerative therapies [76]. MSCs have also been utilized for limb ischemia in both animals and humans with a low benefit so far [169–172]. To improve their potential some researchers have defended their combination with GH. First, as described, GHR have been found in these progenitor cells [173], facilitating the differentiation and growth factors secretion from MSCs by GH. For example, it has been published how MSCs that overexpress Akt improve their actions, increasing the power to repair damaged myocardium despite an infrequent cellular fusion or differentiation [174]. However, MSCs rather than stimulate growth factors secretion, facilitate the release of them

from surrounding tissues [171]. This insight confirms that paracrine mechanisms mediated by MSCs are the authentic players to enhance the survival of existing myocytes, and that stimulating the Akt pathway, we could act on cytokines and growth factors secretion. GH might favor the stimulus of this signaling pathway in MSCs. In fact, knockout mice for GH receptor (GHRKO) show how MSCs tend to differentiate into adipocytes, partially losing their potential, both in bone marrow and peripheral tissues. Wnt/β-catenin signaling pathway seems to increase when GH is present, suggesting that it has a role in the modulation of MSCs fate by GH [175]. This fact has also been confirmed in MSCs of human trabecular bone. Human bone marrow MSCs express GHR and respond to GH via JAK2/STAT5 intracellular signaling [176]. These findings support the idea that MSCs activity might be modulated with independence of the origin of these cells, and that GH can be a true stimulator for them. Thus, since MSCs are essential for vasculogenesis, they could be crucial during adult arteriogenesis given the similarity between both processes, and GH could be of help to increase the potential and modulate the action of MSCs, or even facilitating their differentiation in SMCs favoring collateral growth.

3.7. GH and extracellular matrix (ECM)

Collateral remodeling is the final step for arteriogenesis, occurring mainly in the media and adventitia layers. SMCs and fibroblasts play the main role in this phase, in which the external elastic lamina and the adventitia elastin are degraded by proteolytic enzymes such as metalloproteinases (MMP) and plasmin to make room for the growing vessel. FGF-2 or IGF-I, among others, will trigger the maturation and proliferation of fibroblast and SMCs [177]. Cell migration is key for both angiogenesis and arteriogenesis. However, migrating cells need of a scaffold to do it. For this, the extracellular matrix (ECM) plays a pivotal role, and GH can regulate it [178,179]. For instance, when the hormone is administered to cultured human SMCs it produces a direct and dose-dependent increase of hyaluronic acid and chondroitin [179]. That is, the remodeling process takes place by dynamic restructuring of the ECM with degradation and synthesis, and GH, on a hand, can act as a mitogenic factor, also favoring the release of growth factors and migration of a major players in this process such as SMCs, fibroblasts, ECs or the same macrophages, in the vascular wall. In a study with cultured murine thymic endothelioma cells (tEnd.1), the treatment with GH for 24 h induced an increase in the production of fibronectin and laminin from these ECs, as compared to the control, also rising the expression of ECM receptors for fibronectin and laminin, and the migratory activity of the aforementioned cell line [178]. On the other hand, activation of macrophages in the vascular wall will facilitate MMP production, influencing ECM degradation. All these data confirm the ability of GH to influence ECM, playing an important role for vessels remodeling.

3.8. GH and NO-independent vascular tone: The role of sympathetic system

Sympathetic innervation seems to be necessary for stabilizing vascular wall tone and cells phenotype, since in sympathectomized vessels both SMCs and fibroblasts increase in numbers, with collagen alterations (collagen III upregulation and collagen IV downregulation). This supports the hypothesis that the autonomic system participates in vascular homeostasis [180]. In GHD patients a marked increase in sympathetic activity has been found [181], but it tends to be reversed after GH replacement therapy [182], suggesting that the hormone may regulate central sympathetic activity, affecting vascular peripheral resistance. When sympathetic activity is increased collaterals will suffer an intimal thickening that diminishes SSF and collateral enlargement after ischemia [180]. An increased vasomotor tone has been described in skeletal muscle arterioles in diabetic patients with neuropathy, and a higher α-adrenergic tone has been found in the iliac artery of diabetic animals [183,184]. Thereupon, proper innervation plays an important role in the development and remodeling of blood vessels, although the exact mechanism of impaired arteriogenesis, when altered, is still poorly understood.

That GH is related to the autonomous system has not only been described in GHD patients. In healthy humans, since GH release after GHRH administration has been found associated with sympathetic activation and baroreflex resetting in a microneurographic study of muscle sympathetic nerve activity, as compared to placebo group at rest, whereas blood pressure and heart rate were not

altered [185]. Therefore, GH is related to sympathetic system both during physiological and pathological situations of GH secretion.

3.9. GH and midkine (MDK) relation should be investigated

Midkine (MK), a heparin-binding growth factor that seems to play an important role in arteriogenesis by mediating NO synthases and increasing the bioavailability of VEGFA [186], has also been related with GH, as expression of MK and its receptors have been detected in somatotrophs of both embryonic and adult rats, favoring the development of pituitary gland [187] and acting as a regulator of its function in the adult. MK seems to be secreted by follicle stimulating cells, controlling GH production from GH cells in a paracrine way via protein tyrosine phosphatase receptor-type Z (Ptprz1) [188]. MK is involved in the process of mechanosensing to SSF, as its receptor has an identical structure of sensors for laminar shear stress and induces a very similar signal cascades [189]. A mechanosensing mechanism has been proposed for collateral enlargement with the main participation of several molecules such as PECAM-1, VE-Cadherin or VEGFR2/KDR [190], to which, in our opinion, GH/IGF-I should be added, as it has been demonstrated at the beginning of the text. Nevertheless, from the findings of the GHAS trial, GH significantly increases KDR in ischemic areas, possibly participating in mechanical signals and vasopermeability. An altered hypertrophic vascular remodeling has been seen in MK-deficient mice, due to the reduction in ECs proliferation, although medial and adventitial layers remain normal [191]. This means that ECs proliferation is a major actor for correcting vascular growth, and, as described before, GH is mainly involved in ECs proliferation, participating from this point of view in this mitogenic and proliferative process.

Given that GH is a major regulator of eNOS expression and NO production in ECs for vasodilation, that typically increases VEGF and KDR, and as a consequence of its participation in vascular homeostasis, it is tempting to speculate that both MK and GH might work together to maintain vascular homeostasis, and also to activate vascular enlargement when an ischemic condition is present. MK could promote vasodilation indirectly by increasing the bioavailability of GH in ECs [189]. However, this hypothesis is not yet proved and needs confirmation.

3.10. GH and Klotho: the perfect combination?

Of interest is the special relationship between Klotho, a molecule that is gaining relevance in recent years, and GH. A comprehensive review of the relation between GH and Klotho has been perfectly described [16,192]. Here the intention is just to highlight the importance of this relationship for arteriogenesis. As is known, Klotho is a type of transmembrane full-length protein found mainly in the kidneys that can act remotely generating a circulating form by two processes: proteolytic cleavage of the membrane protein by secretases, called soluble Klotho (sKlotho) [193]; or the secreted Klotho after mRNA splicing, the latter being the most important source of total circulating Klotho. This form can work as a hormone regulating the functions of cells that lack this protein, such as ECs and arterial SMCs [194]. Receptors for Klotho in these tissues and the exact mechanisms of actions have not been properly stablished. It is known that circulating Klotho increases both GH production by the anterior hypophysis [195], and the local production of the hormone by the endothelium, and besides inhibits the negative feedback of IGF-I on GH secretion [196]. Both molecules are essential for vascular homeostasis, but also when this process is altered. It is though that the lack of Klotho production in chronic renal insufficiency is the reason for aging and calcification of the vessels wall, even in collaterals, and in consequence, it impairs atherosclerosis and arteriogenesis. This fact is related to the phosphate levels, since Klotho regulates the calcium entry into cells and inhibits the action of phosphate on the vascular wall. In fact, mice overexpressing Klotho have a longer life expectancy due to the mentioned effect by Klotho and its anti-IGF-I action [197]. Conversely, animals overexpressing GH seem to live less because of the increase in IGF-I [198]. Thus, Klotho stimulates GH while blocks IGF-I, eliminating the deleterious effects of the latter. But GH also exerts influences on Klotho levels, as patients with acromegaly show increased levels of Klotho and they show a downregulation when the GH-produced pituitary adenoma is removed [192].

Circulating Klotho could be important in physiological states, but also in pathological ones, since mice with Klotho deficiency show vascular hyperpermeability. This molecule seems to be necessary for the control of VEGF action on the vascular permeability. At the level of the endothelium, Klotho

facilitates the association of both KDR and transient receptor potential canonical calcium channel 1 (TRPC-1) promoting their cointernalization and regulating calcium entry to maintain homeostasis. Thus, KDR is needed by Klotho to accomplish its actions [199]. GH, that, as demonstrated before, increases KDR in ischemic muscle of the leg, could collaborate with Klotho this way facilitating this action. But the most attractive action of Klotho for arteriogenesis is that it also participates in the regulation of vascular tone, as compensates the vasoconstrictor action of some factors such as phosphates or FGF23 by increasing the production of NO [200], an action probably mediated by local GH.

Curiously, in vitro studies show how Klotho can act on vascular wall. When administered alone, this molecule seems to favor vascular contraction in SMCs of aortic rings samples from mice, increasing ROS levels both in SMCs and ECs, as also occur when SMCs are in the presence of FGF23 and phosphates. However, when SMCs pretreated with FGF23 or phosphates are exposed to Klotho the relaxation phenomenon predominates, while ROS levels remain elevated. This is an effect mediated by indirect NO production by Klotho from ECs stimulating both eNOS and iNOS [200]. This was confirmed by the fact that when the endothelium was removed this effect disappeared, highlighting the involvement of the endothelium. This supports the aforementioned finding that small levels of redox stress contribute to the regulation of vascular homeostasis, as eNOS is sensitive to ROS, and also the complex action that Klotho has depending of its environment, as it happens with GH. The other important message is the main role of the ECs in the control of vascular tone and SMCs actions. Thus, Klotho seems to benefit from interacting with other growth factors in each specific tissue, helping in the regulation of vascular tone in physiological conditions, and, necessarily, has to participate in arteriogenesis in pathological states such as ischemia, although this aspect has to be confirmed, in the same way that it should be done for its collaboration with GH for it.

4.- Conclusions

Vascular homeostasis critically depends on the physiological response of endothelial cells to the blood supply and the appropriate redox balance. The endothelium releases many factors to control vascular tone, adhesion of circulating blood cells, smooth muscle cells proliferation and inflammation.

Why should GH be considered a promising therapeutic agent for neovascularization? The GH/PRL/PL family regulates the physiological growth and regression of blood vessels in the female reproductive organs, and this fact strongly support their vascular role in neovascularization. There is no doubt about the fact that the GH/IGF-I axis has to be an important factor in the former process, both in physiological and pathological states, as many evidences have underlined. This axis suffers an important decline with aging, mainly affecting GH secretion. Considering that most patients with ischemic injuries are elderly, GH therapy could be considered of help to mitigate symptoms and improve vascularization.

However, the information concerning the regulation of neovascularization by proangiogenic hormones as GH is insufficient, since few physiological or pathological conditions have been studied deeply, reporting some exceptions and contrary effects, as a consequence, at least in part, of different animal models of ischemia, type of tissue analyzed, disease status, hormone dose or follow-up time. These effects also depend on the relative contribution of local production of hormones, or on the hormonal cleavage by proteases or clearance by kidneys. Surprisingly, the data is also limited about endogenously produced antiangionenic substances that might be overexpressed in chronic states such as ischemia and that could act with a harmful effect on GH actions.

The role of redox balance in arteriogenesis and how GH could aid mitigating it have been analyzed. We have also proposed the possibility that GH and IGF-I could be part of those mitogenic factors secreted by endothelial cells in response to shear stress forces. The large number of connections that both molecules have with cytokines, hormones and cells involved in neovascularization reinforce their role in this process. Finally, as a novelty it has been presented in this review some insights from the GHAS trial in patients with critical limb ischemia that can help to understand the action of GH dealing with ischemia. Nevertheless, the molecular results of this clinical study still need to be confirmed.

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